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A c-type cytochrome and a transcriptional regulator responsible for enhanced extracellular electron transfer in *Geobacter sulfurreducens* revealed by adaptive evolution

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Summary

The stimulation of subsurface microbial metabolism often associated with engineered bioremediation of groundwater contaminants presents subsurface microorganisms, which are adapted for slow growth and metabolism in the subsurface, with new selective pressures. In order to better understand how *Geobacter* species might adapt to selective pressure for faster metal reduction in the subsurface, *Geobacter sulfurreducens* was put under selective pressure for rapid Fe(III) oxide reduction. The genomes of two resultant strains with rates of Fe(III) oxide reduction that were 10-fold higher than those of the parent strain were resequenced. Both strains contain either a single base-pair change or a 1 nucleotide insertion in a GEMM riboswitch upstream of GSU1761, a gene coding for the periplasmic *c*-type cytochrome designated PgcA. GSU1771, a gene coding for a SARP regulator, was also mutated in both strains. Introduction of either of the GEMM riboswitch mutations upstream of *pgcA* in the wild-type increased the abundance of *pgcA* transcripts, consistent with increased expression of *pgcA* in the adapted strains. One of the mutations doubled the rate of Fe(III) oxide reduction. Interruption of GSU1771 doubled the Fe(III) oxide reduction rate. This was associated with an increased in expression of *pilA*, the gene encoding the structural protein for the pili thought to function as microbial nanowires. The combination of the GSU1771 interruption with either of the *pgcA* mutations resulted in a strain that reduced Fe(III) as fast as the comparable adapted strain. These results suggest that the accumulation of a small number of beneficial mutations under selective pressure, similar to that potentially present during bioremediation, can greatly enhance the capacity for Fe(III) oxide reduction in *G. sulfurreducens*. Furthermore, the results emphasize the importance of the c-type cytochrome PgcA and pili in Fe(III) oxide reduction and demonstrate how adaptive evolution studies can aid in the elucidation of complex mechanisms, such as extracellular electron transfer.

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Introduction

A frequently proposed strategy to immobilize soluble metal contaminants in groundwater is to add organic electron donors to promote the reductive precipitation of the metals (Lloyd and Lovley, 2001). For example, adding organic compounds to organic-poor subsurface sediments typically promotes the growth and activity of Fe(III)-reducing *Geobacter* species (Snoeyenbos-West *et al*., 2000; Holmes *et al*., 2002; Anderson *et al*., 2003; Istok *et al*., 2004; North *et al*., 2004; Chang *et al*., 2005), which primarily obtain energy to support growth from Fe(III) reduction (Finneran *et al*., 2002), but will also reduce contaminant metals such as U(VI) (Lovley *et al*., 1991), V(V) (Ortiz-Bernad *et al*., 2004) and Co(III) (Caccavo *et al*., 1994). These highly unnatural conditions may impose selective pressure for strains that have the ability to reduce Fe(III) oxides much more rapidly than is required in organic-poor sediments.

The oxidation of organic compounds coupled to Fe(III) oxide reduction in *Geobacter* species is a complex process, which probably requires direct contact between the cells and the Fe(III) oxides (Nevin and Lovley, 2000; Childers *et al*., 2002). In *Geobacter sulfurreducens* reducing equivalents from the TCA cycle are oxidized by the NADH-dependent dehydrogenase (Galushko and Schink, 2000; Butler *et al*., 2009). Generated protons are returned

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to the cytoplasm for ATP generation (Mahadevan *et al*., 2006). Electrons are transferred to the menaquinones pool and then to periplasmic *c*-type cytochromes such as PpcA (Lloyd *et al*., 1999; 2003; Galushko and Schink, 2000; Butler *et al*., 2009). Multiple outer membrane proteins, including several *c*-type cytochromes, a porin (OmpJ), a couple of multi-copper proteins (OmpB, OmpC) and pili-forming PilA, are known to be necessary for the electron transfer to the final acceptor (Lovley *et al*., 2008). Moreover, chemotaxis in *Geobacter metallireducens* facilitates access to Fe(III) oxide (Childers *et al*., 2002). It seems feasible that if beneficial mutations rendering *Geobacter* species more capable in one or more of these Fe(III) oxide reduction steps arise during bioremediation, this may provide that strain with a selective advantage.

Laboratory adaptive evolution experiments allowing manipulation of environmental variables are a good strategy to illustrate the plasticity of microbial genomes upon generation of a selective pressure (Elena and Lenski, 2003). When combined with whole-genome resequencing, adaptive evolution is also likely to provide new information about the physiology of a given organism and the associated regulatory mechanisms (Friedman *et al*., 2006; Herring *et al*., 2006; Velicer *et al*., 2006; Gresham *et al*., 2008; Conrad *et al*., 2009). The purpose of this study was to determine whether *G. sulfurreducens* could be adapted for faster growth on Fe(III) oxide, and if so, to elucidate what mutations accumulated that might promote Fe(III) oxide reduction.

Results and discussion

Adaptation of strains for faster Fe(III) oxide reduction

Two strains of *G. sulfurreducens* capable of reducing insoluble Fe(III) oxide significantly faster than wild-type cells were produced with slightly different approaches (Fig. 1). Strain V1 was isolated from a culture that had been continuously transferred in Fe(III) oxide medium approximately once a week over a 2-year period. In an attempt to recreate V1 under more rigorously defined conditions, the *G. sulfurreducens* PCA genomic strain was cultivated with Fe(III) oxide as the electron acceptor and transferred (10% inoculum) into fresh Fe(III) oxide medium when Fe(II) concentrations reached approximately 25 mmol l^{-1} in order to continuously transfer the culture during the most active growth phase on Fe(III) oxide. After 27 transfers over a 20-months period, an isolate, designated strain V2, was obtained on solidified Fe(III) oxide medium.

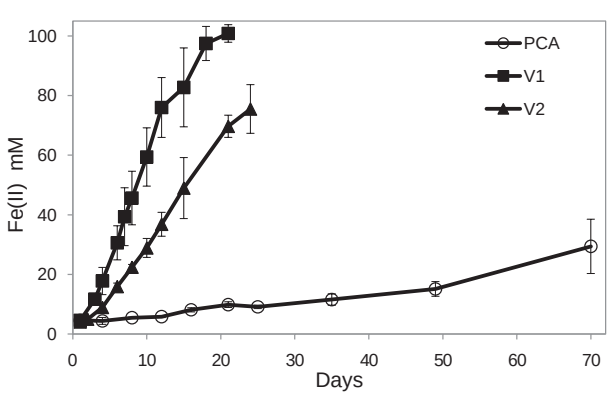

Fig. 1. Reduction of Fe(III) oxide by the *G. sulfurreducens* genomic strain PCA, adapted strains V1 and V2. Each curve is the mean of at least three independent replicate cultures.

The V1 and V2 strains had lag periods of, respectively, 24.4 ± 3.1 h and 25.5 ± 0.1 h with soluble Fe(III) citrate as the electron acceptor. This is significantly shorter than the lag period of 38.7 h ± 4.0 observed with strain PCA. With respective rates of soluble Fe(III) reduction of 1.4 mM Fe(II)/h ± 0.0 and 1.6 mM Fe(II)/h ± 0.1, V1 and V2 strains are slightly faster than PCA [1.2 mM Fe(II)/h ± 0.1]. Like Fe(III) oxide, Fe(III) citrate is reduced on the outer surface of the cell (Coppi *et al*., 2007) and the outer-membrane *c*-type cytochrome OmcB is required for optimal reduction of both forms of Fe(III) (Leang *et al*., 2003). However, several of the redox active, outer-surface proteins required for optimal Fe(III) oxide reduction, such as the *c*-type cytochromes OmcS and OmcE (Mehta *et al*., 2005) and the multicopper protein OmpB (Mehta *et al*., 2006), as well as the electrically conductive pili known as microbial nanowires (Reguera *et al*., 2006), are not required for Fe(III) citrate reduction.

Strain V1 did not grow on fumarate, due to a mutation in the fumarate transporter DcuB (see below). When a copy of *dcuB* was introduced on the chromosome, V1 grew on fumarate, but with a doubling time of 12.1 h ± 0.3, which is approximately twice that of PCA (5.9 h ± 0.3). V2 grew at a similar slower rate of 11.8 h ± 0.8. In contrast to Fe(III) citrate and Fe(III) oxide, fumarate is reduced inside the cell by the fumarate reductase, an enzyme bound to the cytoplasmic membrane (Galushko and Schink, 2000; Butler *et al*., 2006). Thus, these results suggested that there had been a specific adaption in V1 and V2 for faster rates of extracellular electron transfer, rather than an overall adaptation for faster rates of respiration.

Mutations associated with faster Fe(III) oxide reduction

The potential that mutations in V1 and V2 might be associated with the enhanced capacity for Fe(III) oxide reduction was evaluated by genome resequencing using Nimblegen CGS and Illumina sequencing. Nimblegen CGS is a method comparing the hybridization of mutated DNA to microarray probes to the hybridization of non-mutated DNA (Albert *et al*., 2005). Illumina technology generates random short sequence reads by way of

Table 1. Mutations found in *G. sulfurreducens* adapted strains V1 and V2.

	Adapted strain mutation[a,b]	
Mutated gene	V1	V2
pgcA (GSU1761): c-type triheme cytochrome	Insertion of a G; 82 bp upstream of *pgcA* start codon in the rho-independent terminator of a GEMM riboswitch[c]	C ⇒ T; 99 bp usptream of *pgcA* start codon in the rho-independent terminator of a GEMM riboswitch
GSU1771: transcriptional regulator	IS element; interruption of GSU1771 after 93 bp by ISGsu4[d]	3 bp in-frame deletion; loss of ile-22
GSU2622: HAMP/GAF/HD-GYP protein	T ⇒ C; val-16 to ala substitution in the predicted signal peptide	
dcuB (GSU2751): fumarate/ succinate antiporter	Insertion of a T; shift in the protein sequence after Ser-140 and stop codon after 217 amino acids (unadapted protein has 442 amino acids)	
GSU3261: Response regulator	12 bp in-frame deletion; loss of ala-82, asp-83, gln-84 and arg-85 in the single REC domain[d]	

a. Mutation was found in two isolates of each adapted strain.
b. Adapted strains were resequenced by NIMBLEGEN and Illumina and mutations were confirmed by Sanger sequencing.
c. Mutation detected only by Illumina.
d. Mutation detected only by Nimblegen CGS.

sequencing by synthesis using labelled reversible terminators (Tettelin and Feldblyum, 2009). Sanger sequencing was used to confirm the existence of the mutations detected by Nimblegen CGS and/or Illumina sequencing.

V1 contained five confirmed mutations whereas V2 had only two mutations (Table 1). Some of these mutations were detected only by one of the two resequencing methods (Table 1). The two genes that were mutated in V2 were also mutated in V1. One of the genes that were mutated in both strains was GSU1761 (designated *pgcA* for periplasmic *GEMM-regulated cytochrome A*), which is predicted to be a 1536 bp monocistronic gene (Krushkal *et al*., 2007) coding for a *c*-type triheme cytochrome. The PgcA protein has been detected in proteomic studies and was primarily recovered from the periplasmic cell fraction (Ding *et al*., 2006). PgcA was also more abundant in cells grown on insoluble Fe(III) oxide than soluble Fe(III) citrate (Ding *et al*., 2008). Homologues of PgcA are found in several other *Geobacter* species able to grow with insoluble Fe(III) oxide including *Geobacter bemidjiensis* [Gbem_1881] and *Geobacter daltonii* [Geob_3176]. *pgcA* contains a GEMM (genes related to the environment, membranes and motility) riboswitch sequence between the predicted RpoD-dependent promoter and the start codon (Table 1) (Weinberg *et al*., 2007). GEMM riboswitches sense cyclic-di-GMP (Sudarsan *et al*., 2008). An increase in cyclic-di-GMP in *E. coli* results in the upregulation of *lacZ* under the control of the *pgcA*-associated GEMM riboswitch (B.C. Kim, pers. comm.). In both V1 and V2 the mutation in *pgcA* was upstream of the start codon, within the riboswitch region. However, the mutations were different with insertion of a G 82 bp upstream of the start codon in V1 and a transition of a C to a T 99 bp usptream of the start site in V2.

The other gene that was mutated in both V1 and V2 was GSU1771 (Table 1). This is a 648 bp monocistronic gene (Krushkal *et al*., 2007) encoding a 215-amino-acid *Streptomyces* antibiotic regulatory protein (SARP)-like protein. SARP proteins have previously been described in *Streptomyces* species in which they regulate secondary metabolite biosynthesis (Bibb, 2005). Similar to previously described SARP regulators, GSU1771 possesses a N-terminal DNA-binding domain (34–112 AA) similar to the one found in *E. coli* OmpR and a C-terminal bacterial transcriptional activator domain (119–183 AA) (Wietzorrek and Bibb, 1997; Yeats *et al*., 2003). Homologues of GSU1771 are found in two other *Geobacter* species, *G. metallireducens* [Gmet_1852] and *G. uraniireducens* [Gura_1936]. In V1, GSU1771 is disrupted by an ISGsu4 insertion element containing a gene coding for a transposase. In V2, GSU1771 contains an in-frame deletion of three nucleotides resulting in the lost of an isoleucine at position 22. This residue is located in a poorly conserved region among SARP regulators.

Three other genes, GSU2622, *dcuB* (GSU2751) and GSU3261, were also mutated in V1 (Table 1). GSU2622 encodes for a HAMP/GAF/HD-GYP protein with a putative haem-binding site (Londer *et al*., 2006). GSU3261 is a response regulator with a single REC domain. The functions of these two proteins are unknown. DcuB is a fumarate/succinate antiporter required for growth of strain PCA on fumarate (Butler *et al*., 2006). It is also known to participate in the regulation of the expression of fumarate respiration genes in *E. coli* (Six *et al*., 1994; Kleefeld *et al*., 2009).

Gene expression profiling of V1 and V2

In order to better define the possible mechanisms for faster Fe(III) oxide reduction by the adapted strains, gene expression in strains V1 and PCA was compared with whole-genome DNA microarrays. The poor growth of

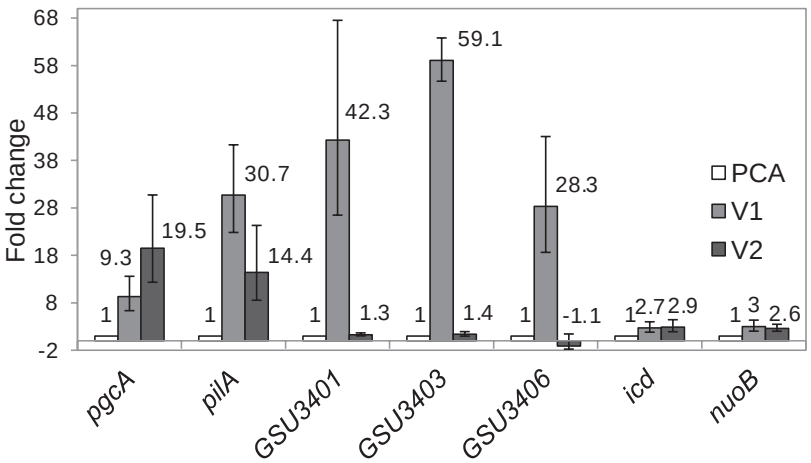

Fig. 2. Quantitative RT-PCR assay of genes upregulated in both ferric citrate and fumarate V1 microarrays. Fold change in V1 and V2 compared with *G. sulfurreducens* PCA during growth on ferric citrate. Each value is the mean of at least three replicates.

strain PCA on Fe(III) oxide prevented recovery of sufficient mRNA for analysis of transcript abundance during growth on Fe(III) oxide. Therefore, gene expression during growth on Fe(III) citrate and fumarate was compared. With both alternative electron acceptors the gene with the highest increase in transcript abundance in V1 versus PCA was *pgcA* (Table S1). qRT-PCR analysis confirmed higher transcript abundance of *pgcA* in V1 and V2 than in PCA (Fig. 2).

The microarray analysis revealed that GSU1496 and GSU1497 were also substantially more highly expressed in V1 than PCA (Table S1). These two genes are in an operon (Krushkal *et al*., 2007). GSU1496 encodes PilA, the structural protein for the Type IV pilin of *G. sulfurreducens*. Previous studies have demonstrated that these pili are required for optimal Fe(III) oxide reduction (Reguera *et al*., 2005) as well as electron transfer to electrodes (Reguera *et al*., 2006; Nevin *et al*., 2009). The pili appear to be electrically conductive and it has been suggested that they may function as 'microbial nanowires', serving as a conduit for electron transfer to insoluble, extracellular electron acceptors (Reguera *et al*., 2005; 2006). The function of GSU1497 has not yet been determined, but is presumed to also be related to pilin function. Higher transcript abundance of GSU1496 in V1 and V2 was confirmed with qRT-PCR (Fig. 2).

Other highly upregulated genes in V1 detected with microarray analysis included the predicted operon GSU3401-3402 and the predicted monocistronic gene GSU3403 (Krushkal *et al*., 2007) (Table S1). These genes are also more highly expressed in *G. sulfurreducens* growing on the surface of graphite electrodes serving as the electron acceptor than in cells growing on the same graphite surface, but with fumarate serving as the electron acceptor (Nevin *et al*., 2009), suggesting that the function of these genes is related to extracellular electron transfer. The functions of the proteins encoded by these three genes have not yet been investigated, but GSU3401 is predicted to encode an amino acid-binding protein. GSU3406, which encodes another putative amino acid binding protein, also had higher transcript abundance in V1 (Table S1) and was also previously found to be more highly expressed in current-producing cells (Nevin *et al*., 2009). The higher expression of GSU3401, GSU3403 and GSU3406 in V1 was confirmed with qRT-PCR (Fig. 2). However, their expression levels in V2 were comparable to that of PCA (Fig. 2). This suggested that high expression of these genes may not be essential for primary enhancement in Fe(III) oxide reduction observed in V1, but their enhanced expression in V1 might be one of the reasons why V1 reduces Fe(III) oxide faster than V2.

There were other genes with somewhat lesser increases in transcript abundance in V1 versus PCA. These genes encode TCA cycle enzymes or subunits of the NADH-dependent dehydrogenase (Table S1). qRT-PCR confirmed that the expression of *icd,* which is predicted to be in an operon with *mdh* (Krushkal *et al*., 2007), and *nuoB*, which is in the same operon as all the other subunits of the NADH-dependent dehydrogenase, were slightly more highly expressed in V1 and V2 (Fig. 2). These results suggest a potential for higher rates of respiration in V1 and V2 than in PCA, but, as noted above, faster respiration was not evident during growth with fumarate as the electron acceptor, even if these genes were more highly expressed in fumarate-grown cells.

Faster Fe(III) oxide reducing strains recreated with genetic manipulations

In order to evaluate the potential roles of the mutations in *pgcA* and GSU1771 in enhanced Fe(III) oxide reduction, three constructions mimicking the V1 *pgcA*, the V2 *pgcA* or the V1 GSU1771 mutations were made. To recreate the single base-pair insertion or the C ⇒ T transition, respec-

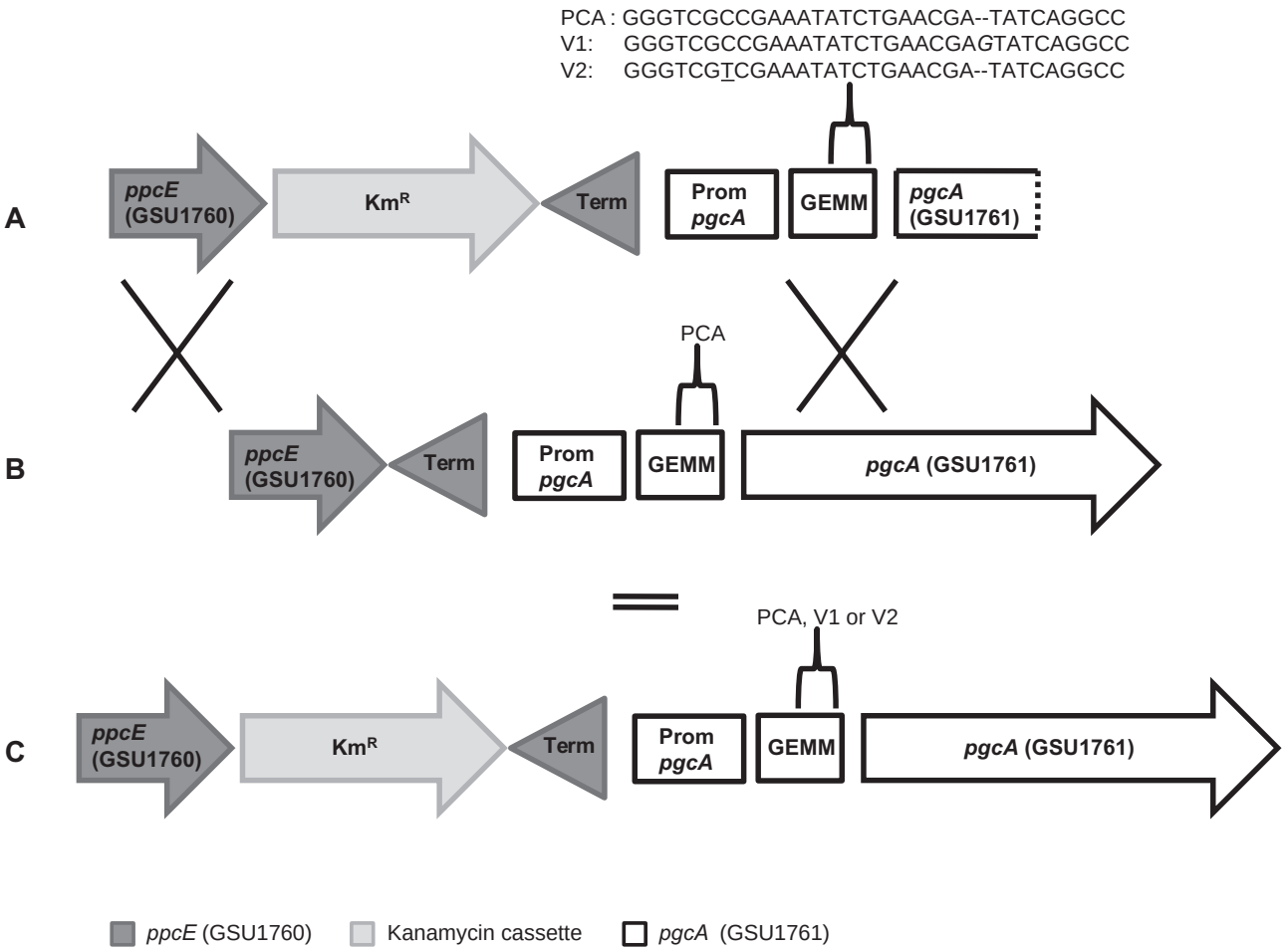

Fig. 3. Introduction of mutations found in V1 and V2 *pgcA* GEMM riboswitches into *G. sulfurreducens* PCA chromosome. A kanamycin cassette was included in the middle of PCR constructs (A) containing in 5′ the complete PCA *ppcE* gene and in 3′, PCA, V1 or V2 *ppcE* rho-independent terminator (Term), *pgcA* promoter region (Prom), *pgcA* GEMM riboswitch and the beginning of *pgcA* coding sequence. The base pair inserted in V1 *pgcA* GEMM riboswitch is in italic whereas the transition found in V2 *pgcA* GEMM riboswitch is underlined. Following electroporation, the linear PCR constructs were incorporated by homologous recombination in PCA chromosome (B). This process results in three strains containing a kanamycin cassette and either the PCA, V1 or V2 *pgcA* GEMM riboswitch sequence (C).

tively, found upstream of *pgcA* in V1 and V2, genetic constructs bearing the desired mutations and including a kanamycin cassette as a selective marker located between the stop codon and the predicted rho-independent terminator of GSU1760 (*ppcE*) were assembled (Fig. 3). To mimic the transposon-interrupted version of GSU1771 found in V1, this gene was disrupted by a spectinomycin cassette. The V1 *pgcA*, V2 *pgcA* and GSU1771::Spec^R constructs were introduced alone or in combination in PCA.

Introducing the V1 *pgcA* mutation into PCA had no impact on Fe(III) oxide reduction beyond that of a control in which just a kanamycin resistance cassette was inserted between the stop codon of *ppcE* (GSU1760) and its associated rho-independent terminator, leaving a wild-type *pgcA* intact (Fig. 4A–C). This was despite the fact that this mutation resulted in a significant increase in *pgcA* transcript abundance compared with the control (Fig. 4D). Introducing the V2 *pgcA* mutation in PCA, increased *pgcA* transcript abundance more than the V1 *pgcA* mutation and resulted in an approximate doubling of the rate of Fe(III) oxide reduction over the control (Fig. 4A–C).

Disrupting GSU1771 in the control strain (Fig. 4A–C) or wild-type PCA (data not shown) also nearly doubled the rate of Fe(III) oxide reduction. The most dramatic increases in Fe(III) oxide reduction were in strains that combined the GSU1771 disruption with either of V1 or V2 *pgcA* mutations (Fig. 4B). When the lag period that was also observed in the control strain is discounted these strains reduced Fe(III) oxide as rapidly as V2 (Fig. 4C). One clear impact of the disruption of GSU1771 was a dramatic increase in expression of *pilA* (Fig. 4D).

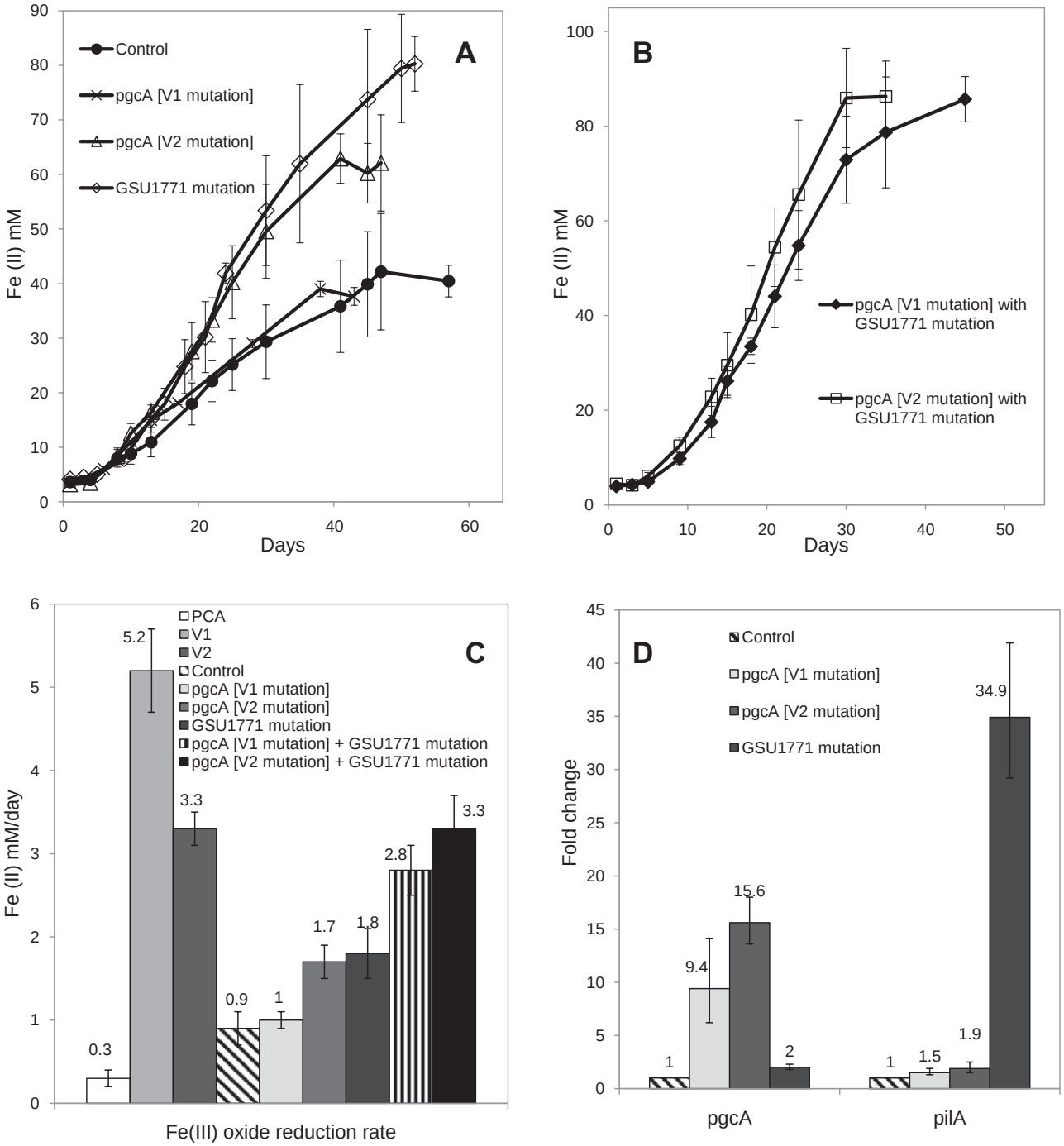

Fig. 4. Fe(III) oxide reduction and gene expression in strains mimicking strains V1 and V2. All strains contain the kanamycin cassette upstream of GSU1761 that is present in the control strain. Fe(III) oxide reduction in control strain and strains with single mutations (A) or in double mutants (B). Each curve is the mean of at least three independent Fe(III) oxide reduction experiments. Summary of Fe(III) oxide reduction rates of all strains in this study (C). Impact of mutations on expression of *pgcA and pilA* measured by qRT-PCR compared to the control strain during growth on Fe(III) citrate (D). The qRT-PCR results are from at least three replicates for each strain.

Implications

This study demonstrates that selective pressure for rapid Fe(III) oxide reduction can result in the accumulation of mutations that enhance rates of Fe(III) oxide reduction. It seems likely that the practice of introducing high concentrations of organic electron donors to stimulate dissimilatory metal reduction for *in situ* bioremediation of

contaminated groundwaters might apply a similar selective pressure (Anderson *et al*., 2003; Ortiz-Bernad *et al*., 2004). Thus, in addition to the more commonly considered selection from pre-existing strains that is associated with changes in environmental conditions, new strains, which have accumulated beneficial mutations, might also emerge that might influence the speed and/or effectiveness of bioremediation. As the costs for large-scale sequencing of environmental genomic DNA continue to decline, it should be feasible to evaluate this possibility during the bioremediation process.

The mutations that accumulated in *G. sulfurreducens* in response to selective pressure for more rapid Fe(III) oxide reduction provide some insight into some of the cellular components that may play an important role in the reduction of Fe(III) oxides. For example, the increased expression of the gene for the pilin structural protein, PilA, that was associated with the mutation in GSU1771, and the enhanced Fe(III) oxide reduction by the mutant strain, provides further evidence for the suggestion that the pili of *G. sulfurreducens* are important conduits for extracellular electron transfer (Reguera *et al*., 2005; 2006; Nevin *et al*., 2009). Further investigation is necessary to describe the mechanism by which GSU1771 changes the expression of *pilA*, but the apparent regulation of *pilA* expression by SARP-like protein suggests a novel role for this family of regulator.

The primary effect of the *pgcA* mutations is to increase the expression of the encoded c-type cytochrome. The results demonstrated that the single base-pair insertion or the C ⇒ T transition found in the *pgcA*-associated GEMM riboswitch of the adapted strains is largely responsible for the upregulation of *pgcA*. The two mutations influenced the level of expression and hence rates of Fe(III) oxide reduction to different levels. The periplasmic location of *pgcA* suggests that it serves as an intermediary in the electron transfer to Fe(III) oxide. Further characterization of this protein seems warranted.

The additive effect of the combined *pgcA* and GSU1771 mutations demonstrates that there are multiple components that influence electron transfer to Fe(III) oxide. The superior Fe(III) oxide capability of the V1 strain might be attributed to one or more of the additional mutations identified in that strain. However, the finding that the introduction of two mutations could significantly increase the capacity for Fe(III) oxide reduction suggests that, despite the apparent complexity of extracellular transfer, it may be possible to rationally design new strains not only for bioremediation, but also for other applications involving extracellular electron transfer, such as removal of iron contaminants from clays (Lee *et al*., 1999), electron transfer to electrodes (Lovley, 2008; Yi *et al*., 2009) or syntrophic degradation of organic compounds (Stams and Plugge, 2009). It is expected that the adaptive evolution approach described here will continue to identify targets for such genetic engineering and strain design.

Experimental procedures

Bacterial strains, growth conditions and lag times

The bacterial strains and plasmids used are listed in Table S2. Appropriate antibiotics were added when necessary. *Escherichia coli* was cultivated in Luria–Bertani medium. *Geobacter sulfurreducens* strains were routinely cultured anaerobically (N_2/CO_2: 80/20) at 30°C in either acetate-fumarate or acetate-Fe(III) citrate medium as previously described (Coppi *et al*., 2001). For growth studies acetate (10 mM) was the electron donor, and fumarate (40 mm), Fe(III) citrate (55 mM) or Fe(III) oxide (100 mmol l^{-1}) was the electron acceptor as previously described (Caccavo *et al*., 1994; Coppi *et al*., 2001). The concentration of Fe(II) in Fe(III)-reducing cultures was measured with the ferrozine assay as previously described (Lovley and Phillips, 1986).

Culture lag time was approximated as the time at which a regression line associated with the period of most rapid Fe(III) reduction reached the initial value of Fe(II) found in the inoculum. In the case of the fumarate growth curves, the regression line followed the exponential phase before getting to the OD600 value obtained immediately after the addition of the inoculum (Zwietering *et al*., 1992).

Adaptive evolution on Fe(III) oxide

Over a period of 2 years, *G. sulfurreducens* PCA was maintained in Fe(III) oxide medium and transferred into fresh medium at irregular intervals when it appeared from the colour of the iron precipitate that about a third of the 100 mmol l^{-1} Fe(III) initially present had been reduced. The adapted strain obtained in this manner was named V1. Strain V1 was isolated using a set of anaerobic roll tubes (Hungate, 1969). Strain V2 was derived from a study in which Fe(III) reduction was quantified by measuring the accumulation of Fe(II). *Geobacter sulfurreducens* PCA cultures were grown in Fe(III) oxide medium, with transfer of a 10% inoculum into fresh medium when approximately 25 mM of the available 100 mM Fe(III) was reduced to Fe(II). This level of Fe(II) in the medium is analogous to the mid-log phase of a growth curve. After 27 continuous transfers on Fe(III) oxide media, isolated colonies were obtained using the same method as V1 (Hungate, 1969). V1 and V2 Isolated colonies were grown up in 2 ml of Fe(III) oxide media, and a second set of roll tubes was made with the isolates, which were then grown up in 2 ml of media again. The entire 2 ml was transferred to a tube containing 10 ml of Fe(III) oxide medium and the purity of the culture was checked with 16S rDNA PCR. The isolates were transferred to 100 ml bottles of acetate-ferric citrate media. The cultures were spun down in 10 ml aliquots and genomic DNA was extracted using Epicentre MasterPure DNA purification Kit (Epicentre Biotechnologies, Madison, WI, USA). The samples were each eluted in a final volume of 50 µl of protease-free water, and the purity of the DNA was checked with a NanoDrop 1000 spectrophotometer (*NanoDrop* Technologies, Wilmington, DE, USA). DNA from two isolates of both V1 and V2 was subjected to whole-genome resequencing.

Whole-genome resequencing

Mutations found in the adapted strains V1 and V2 were detected by two different whole-genome resequencing methods. Genomic DNA was submitted to the Comparative Genome Sequencing (CGS) service of Nimblegen (Roche NimbleGen, Madison, WI, USA) as described previously (Herring and Palsson, 2007). For Illumina sequencing, genomic DNA libraries were constructed following the manufacturer's protocol (Illumina, San Diego, CA, USA). Briefly, genomic DNA was shorn by nebulization. Following end-repair, Illumina adapters were ligated to DNA fragments. To obtain sufficient DNA (≈ 500 ng), limited PCR was performed using DNA fragments between 190 and 220 bp as templates. Cloning and subsequent Sanger sequencing of several clones confirmed the quality of the libraries. Whole-genome resequencing of the libraries was performed with an Illumina GAII sequencer according to the manufacturer's protocol (Illumina). Genome sequence assembly and polymorphism identification was done as previously described (Conrad *et al*., 2009). For Nimblegen or Illumina whole-genome resequencing, the reference sequence was *G. sulfurreducens* PCA AEO17180 (Methe *et al*., 2003). Regions containing potential mutations were amplified by PCR and sequenced using Sanger sequencing.

DNA microarrays

Gene expression was compared between V1 and PCA on acetate (10 mM)-ferric citrate (55 mM) medium and between V1 *dcuB*$^+$ and PCA on acetate (10 mM)-fumarate medium (40 mM). DNA microarrays were hybridized as described previously (Postier *et al*., 2008). Total RNA was extracted from three independent batch cultures of PCA, V1 or V1 *dcuB*$^+$ using the RNeasy midi kit (Qiagen, Valencia, CA, USA). Purified RNA was treated with DNA-free DNASE (Ambion, Austin, TX, USA) to remove contaminating DNA. The ASAP RNA Labeling kit (Perkin Elmers, Boston, MA, USA) was used to label the experimental samples (V1 and V1 *dcuB*$^+$) with cyanin5 (Cy5) and the control samples (PCA) with cyanin3 (Cy3). Control and experimental labelled RNA preparations were washed, fragmented with the Fragmentation Reagent (Ambion), mixed and hybridized to 12 K Arrays (Combimatrix, Mukilteo, WA, USA) per the manufacturer's instructions. The arrays were scanned using a Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA), and analysed using GenePix and Acuity 4.0 software. Assessing arrays quality and statistical analysis were done as previously described (Nevin *et al*., 2009). LIMMA mixed model analysis [Rpackage LIMMA (Smyth, 2004)] was applied to the normalized Log2 expression ratios to identify differentially expressed genes. Tables S3–S6 list genes significantly upregulated (≥ 2-fold) or downregulated (≤ –2-fold). A gene was considered differentially expressed when at least half of its probes had a *P*-value of ≤ 0.005 and a fold change of ≤ –2 or ≥ 2. Microarrays data have been deposited with NCBI GEO under accession number GSE21322.

qRT-PCR

Sequences from all primers used for quantitative RT-PCR are listed in Table S7. Total RNA was extracted with RNeasy Mini kit (Qiagen) from mid-log acetate-ferric citrate or acetate-fumarate cultures. cDNA was generated with the Enhanced Avian First Strand Synthesis Kit (Sigma-Aldrich, St-Louis, MO, USA) using random primers. The SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI 7500 Real-Time PCR System were used to amplify and to quantify PCR products from *pilA*, *icd*, *nuoB*, *hybS*, *pgcA*, GSU3401, GSU3403, GSU3406 and GSU3410. qRT-PCR for these nine genes confirmed both DNA microarrays results. Expression of these genes was normalized with *proC* expression, a constitutively expressed gene in *G. sulfurreducens* (Holmes *et al*., 2005). Relative levels of expression of the studied genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Mutants construction

Sequences from all primers used for construction of mutants are listed in Table S7. The GSU1771 mutant was constructed following a method described previously (Nevin *et al*., 2009). The coding sequence was replaced by a spectinomycin cassette. Primer pair 1771rg1/1771rg2R1 was used to amplify a ≈ 500 bp fragment upstream from GSU1771 and 1771rg3H3/1771rg4 ≈ 500 bp downstream from GSU1771 with PCA genomic DNA as template. rgspF/rgspR primer pair was used to amplify the spectinomycin cassette from pRG5 (Kim *et al*., 2005). PCR products were combined, digested EcoRI/HindIII and ligated with the T4 DNA ligase (NEB, Beverly, MA). The ligation reaction was loaded onto a 1% agarose gel. A ≈ 2.1 kb band was extracted with Qiaquick gel extraction kit (Qiagen) and served as a template for amplification with the 1761rg1/1761rg4 primer pair. The final GSU1771::Spec^r construction was obtained with 1771rg1/1771rg4 primers.

The construction of Km^r *pgcA*$^+$, Km^r *pgcA*[V1] and Km^r *pgcA*[V2] was done as follows: 1759in1/ppcEinXhoI primer pair was used to amplify a ≈ 500 bp fragment going from the end of GSU1759 to a region located between the stop codon of *ppcE* (GSU1760) and its associated rho-independent terminator with PCA genomic DNA as template. ppcEinXbaI3/1761in4 primer pair was used to amplify a ≈ 500 bp fragment going from a region located between the stop codon of *ppcE* (GSU1760) and its associated rho-independent terminator to the beginning of *pgcA* coding sequence with PCA (Km^r *pgcA*$^+$), V1 (Km^r *pgcA*[V1]) or V2 (Km^r *pgcA*[V2]) genomic DNA as templates. KmXhoIf/KmXbaIr primer pair was used to amplify the kanamycin cassette from pBBR1MCS-2 (Kovach *et al*., 1995). Fragments were combined and digested XhoI/XbaI. After ligation and gel extraction, the three final ≈ 2.1 kb constructions were obtained by using 1759in1/1761in4 primer pair.

To introduce a wild-type copy of *dcuB* (GSU2751) in V1, a PCR amplifying *dcuB* and 500 bp of upstream and downstream sequence from PCA was done with the 2751rg1/2751rg4 primer pair. All the PCR constructs described above were cleaned and concentrated using ethanol precipitation (Sambrook *et al*., 1989). Electroporation of the PCR constructs and mutants isolation were performed as previously described (Coppi *et al*., 2001; Lloyd *et al*., 2003). Mutants were verified by PCR and Sanger sequencing.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes upregulated at least twofold in the adapted strain V1[a] with both electron acceptors: ferric citrate and fumarate (*P*-value cut-off ≤ 0.005).
Table S2. Bacterial strains and plasmids used in this study.
Table S3. Genes that were upregulated at least twofold in the adapted strain V1 when ferric citrate is serving as the electron acceptors (*P*-value cut-off ≤ 0.005).
Table S4. Genes that were downregulated at least twofold in the adapted strain V1 when ferric citrate is serving as the electron acceptors (*P*-value cut-off ≤ 0.005).
Table S5. Genes that were upregulated at least twofold in the adapted strain V1 *dcuB*$^+$ when fumarate is serving as the electron acceptors (*P*-value cut-off ≤ 0.005).
Table S6. Genes that were downregulated at least twofold in the adapted strain V1 *dcuB*$^+$ when fumarate is serving as the electron acceptors (*P*-value cut-off ≤ 0.005).
Table S7. Primers used for mutant construction, complementation and qRT-PCR.

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